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(54) **Cedar pollen protein and uses thereof.**

(57) A protein having a molecular weight of 44,000-54,000 daltons, isoelectric point of 8.5-9.2 and specific sugar chain is prepared from a cedar pollen. The protein induces pollenosis and can be suitably used as desensitization agent because it induces immunoglobulin antibody which is effective for desensitization, but does not substantially induce immunoglobulin E antibody, a major factor causative of side effects including anaphylaxis shock. Therefore, the protein can be advantageously used in the treatment, prevention and/or diagnosis of pollenosis.

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The present invention relates to a novel protein which induces pollenosis and to a process to produce the same, as well as to uses thereof as desensitization agent for treating, preventing and/or diagnosing pollenosis.

For ten and several years, the number of patients, who complain about rhinitis and conjunctivitis due to pollenosis in early spring, has been increasing in Japan Pollenosis frequently receives much publicity from the press because the number is significantly increasing and because it occurs in early spring at which a variety of events, festivals and the like are successively held. Therefore, pollenosis has become one of the problems of the public health which could not be ignored.

It is said that pollenosis is a sort of allergies which is mainly induced by allergens present in cedar pollens (the wording "cedar" as referred to in the invention means *Cryptomeria japonica* and plants of the genus *Cedrus*), i.e., cedar pollen allergens. The invasion of such an allergen, dispersed in the air, into the body resulted in the formation of immunoglobulin E antibody specific to the allergen. When the body being in such conditions is re-invaded by cedar pollens, both cedar pollen allergens contained in the invaded pollens and the already formed immunoglobulin E antibody in the body induce an immunoreaction to cause an allergic symptom.

Until now, it is known that at least 2 different types of allergens with different antigenicities are contained in cedar pollens. The one is an allergen, which is now called "*Cry j* I", as reported by H. YASUEDA et al. in *Journal of Allergy and Clinical Immunology*, Vol.71, No.1, Part 1, pp.77-86 (1983), and the other is an allergen, which is now called "*Cry j* II", as reported by M. TANIAI et al. in *FEBS LETTERS*, Vol.239, No.2, pp.329-332 (1988) or by M. SAKAGUCHI et al. in *Allergy,* No.45, pp.309-312 (1990). Usually, cedar pollens contain *Cry j* I and *Cry j* II in a weight ratio of about 50:1 to 5:1, and most of the sera collected from patients with pollinosis are said to react with both *Cry j* I and *Cry j* II. M. SAWATANI et al. reported in *Japanese Journal of Allergology*, Vol.42, No. 6, pp.738-747 (1993) that *Cry j* II exerts the same level of antigenicity as that exerted by *Cry j* I when assayed on the intradermal test (IT) and radioallergosorbent test (RAST).

As described above, several cedar pollen allergens have been isolated and revealed for their properties and characteristics to some extent, and because of this there appears some possibilities to treat and/or prevent pollenosis by administering to human a purified preparation of a cedar pollen allergen for desensitization. Recently, desensitization agents for such a purpose have been proposed: For example, Japanese Patent Laid-Open Nos.156,926/89 and 93,730/91 propose a method to administer to human a conjugate as desensitization agent prepared by conjugating pullulan as polysaccharide to an allergen having a partial amino acid sequence of Asp-Asn-Pro-Ile-Asp-Ser-or Ala-Ile-Asn-Ile-Phe-Asn- at the N-terminal. Since pollenosis-inducing allergens are not restricted to *Cry j* I and *Cry j* II, other pollenosis-related allergens should be urgently isolated and revealed for their properties and characteristics to establish an accurate diagnosis and an effective desensitization-therapy of pollenosis.

In view of the foregoing, the present invention was made. The present invention aims to provide a novel protein which induces pollenosis.

The present invention also aims to provide a method to prepare the protein.

The present invention also aims to provide the use of the protein as desensitization agent.

According to the present invention there is provided a protein having the following physicochemical properties (hereinafter designated as "protein"). In the specification amino acids, sugars, their coupling modes, etc. may be abbreviated and expressed in accordance with the abbreviations specified by International Union of Pure and Applied Chemistry (IUPAC):

(1) Molecular weight

44,000-54,000 daltons on sodium dodecyl polyacrylamide gel electrophoresis (SDS-PAGE);

(2) Isoelectric point (pI)

8.5-9.2 on isoelectrophoresis;

(3) Partial amino acid sequence containing the C-terminal

Possessing a partial amino acid sequence containing the C-terminal as shown by

-Asn-

Gly-Asn-Ala-Thr-Pro-Gln-Leu-Thr-Lys-Asn-

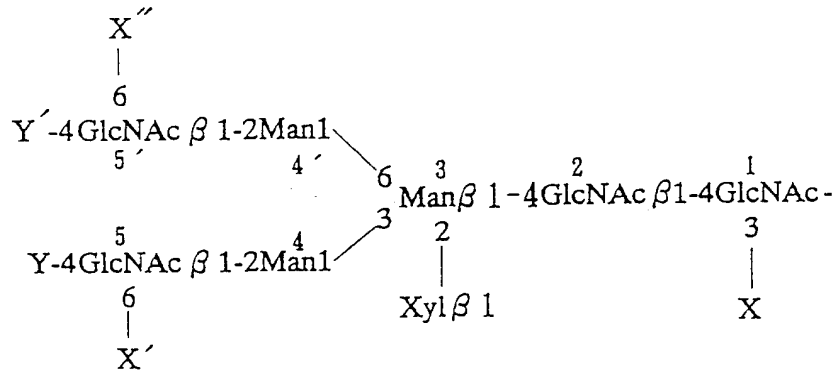
Ala-Gly-Val-Leu-Thr-Cys-Ser-Leu-Ser-Lys-

Arg-Cys;

(4) Sugar content

Containing about 5-20% by weight of a sugar chain having a chemical structure in the molecule as shown by the following chemical formula 1 . Where "X", "X'" and "X''" represent hydroxyl group or Fucα1, and "Y" and "Y'" represent hydroxyl group or Galβ1.

Chemical formula 1:



(5) Ultraviolet absorption spectrum

Exhibiting the maximum absorption spectrum at a wave length around 280 nm;

(6) Solubility in solvent

Soluble in water, physiological saline and phosphate buffer;

(7) Biological activity

Attaching to immunoglobulin E antibody collected from blood of a patient with pollenosis.

Inducing pollenosis; and

(8) Stability

Being inactivated in an aqueous solution (pH 7.2) when incubated at 100 °C for 10 minutes.

Substantially not losing its activity in an aqueous solution (pH 7.2) even when allowed to stand at 4 °C for one month.

A process for preparing a protein is provided, which process comprises extracting a cedar pollen with an aqueous solvent, and recovering a protein from the resultant extract.

The present invention also provides a desensitization agent which contains the protein as an effective ingredient.

The present invention will now be described in further detail, by way of example only, with reference to the following examples.

The protein according to the present invention is a novel substance having specific properties which are entirely different from those of conventional allergens present in cedar pollens.

The present process enables to produce the protein from cedar pollens in a relatively-high yield.

The desensitization agent according to the present invention exerts a considerably-high desensitization activity when administered to mammals including human.

The present invention relates to a novel protein which induces pollenosis. During the research of cedar pollen allergens, we found that there exists an unknown allergen in cedar pollens. We isolated such allergen by employing a combined use of column chromatography as main technique and other purification methods, and investigated the properties and characteristics to reveal that the reality is a protein having different properties and characteristics from those of conventional allergens of cedar pollens.

The following experiments explain the specific properties and characteristics of the present protein:

Experiment 1

Purification of protein

One part by weight of a cedar pollen collected from a male flower of "*omote−sugi*" or *Cryptomeria japonica*, grown in Chiba, Japan, was suspended in and extracted with about 16 parts by weight of an aqueous solution of 0.125 M sodium hydrogen carbonate (pH 8.2) while stirring at 4 °C for one hour, followed by centrifuging the resultant extract to obtain a supernatant. The sediment was treated similarly as above to obtain a supernatant which was then pooled with the above supernatant. To the solution was added 0.1 w/v % "CETAVLON", hexadecyl-trimethylammonium bromide, commercialized by Sigma, Chemicals Co., Louis, USA, and the mixture was centrifuged to obtain a supernatant which was then mixed with ammonium sulfate to give a saturation degree of 80 w/v % to salt out proteinaceous components. The resultant sediment was dialyzed against 50 mM Tris-HCl buffer (pH 7.8) for 10 hours, filtered and fed to a column packed with DEAE-SEPHADEX® which had been previously equilibrated with 50 mM Tris-HCl buffer (pH 7.8), followed by recovering non-adsorbed fractions by feeding 50 mM Tris-HCl buffer (pH 7.8) to the column.

The non-adsorbed fractions were pooled, adjusted to pH 5.0 by the addition of acetic acid, and fed to a column packed with of CM-SEPHADEX® which had been previously equilibrated with 10 mM acetate buffer (pH 5.0). The column was washed by feeding thereto 10 mM acetate buffer (pH 5.0), and fed with an eluant of 0.3 M sodium chloride and 0.1 M phosphate buffer (pH 7.0) to elute proteinaceous components. The fractions containing the proteinaceous components were pooled and fed to a column packed with "MONO-S" which had been previously equilibrated with 10 mM phosphate buffer (pH 5.0), and the column was fed with a liner gradient buffer of sodium chloride increasing from 0 M to 0.5 M in 10 mM Tris-HCl buffer (pH 7.0) to obtain the objective protein contained in fractions with a concentration of 0.1-0.3 M sodium chloride. The fractions were pooled, concentrated in usual manner, lyophilized and used in the following experiments. The yield of the protein was about 0.02% against the material cedar pollen, on a dry solid basis (d.s.b.).

Experiment 2

Physicochemical properties of protein

In this experiment the purified protein obtained in Experiment 1 was studied on its physicochemical properties.

Experiment 2-1

Molecular weight

In accordance with the method as reported by U. K. Lemuli in *Nature*, Vol.227, pp.680-685 (1970), the purified protein was assayed on SDS-PAGE to show a major band in a position corresponding to 44,000-54,000 daltons. The molecular markers used in this experiment were calf serum albumin with a molecular weight of 67,000 daltons, ovalbumin with a molecular weight of 45,000, carbonic anhydrolase with a molecular weight of 30,000 daltons, chymotrypsinogen A with a molecular weight of 25,000 daltons, and cytochrome C with a molecular weight of 12,400 daltons.

A fraction corresponding to 44,000-54,000 daltons was transferred from the gel to a nitrocellulose membrane, and treated with an antibody of anti-cedar pollen allergen derived from mice and an anti-mouse immunoglobulin antibody, derived from goats, labelled with peroxidase from horseradish, to exhibit a distinct immunostaining. The result indicates that the protein is a cedar pollen allergen.

Experiment 2-2

Isoelectric point

The isoelectric point of the purified protein in Experiment 1 was 8.5-9.2 when assayed on isoelectrophoresis.

Experiment 2-3

Partial amino acid sequence containing the C-terminal

Four hundred μg of the purified protein in Experiment 1 was placed in a reaction tube, and dissolved in 300 μl of 6 M guanidine hydrochloride and 10 mM EDTA in 0.5 M Tris-HCl buffer (pH 8.5). The tube was filled with nitrogen gas, and the contents were mixed with adequate amounts of ethyleneimmine and tri-n-butylphosphine. The resultant peptide components were allowed to stand in a light-shielded place overnight to effect aminoethylation. The reaction mixture thus obtained was dialyzed against distilled water, and the dialyzed solution was recovered, lyophilized, dissolved in 300 μl of 0.05 M acetate buffer (pH 4.0), mixed with 15 μg V8 protease, and incubated at 37 °C for 48 hours. Thereafter, the resultant mixture was heated at about 100 °C for 5 min to suspend the enzymatic reaction, and fed to a column packed with anhydrotrypsine agarose which had been previously equilibrated with 0.05 M acetate buffer (pH 5.0) containing 0.02 M calcium chloride, followed by eluting from the column the peptide components adsorbed on the agarose with 0.1 M formic acid.

From the eluate, fractions containing the peptide components were recovered, concentrated, fed to "218TP54", a column for reverse-phase high-performance liquid chromatography commercialized by Vydac, California, USA, which had been previously equilibrated with 0.1 v/v % aqueous trifluoroacetate solution, and eluted with water-soluble acetonitrile containing 0.1 v/v % trifluoroacetate at a flow rate of 0.5 ml/min while the concentration of acetonitrile was increasing at a rate of one v/v % per minute and monitoring the eluate at a wave length of 214 nm. From the resultant eluate containing the peptide components were recovered, concentrated and analyzed for their amino acid sequences on "MODEL 473A", an amino acid sequencer commercialized by Applied Biosystems, Inc., Foster City, USA, to reveal that the protein has a partial amino acid sequence as shown by

-Asn-Gly-Asn-Ala-Thr-Pro-Gln-Leu-Thr-Lys-Asn-Ala-Gly-

Val-Leu-Thr-Cys-Ser-Leu-Ser-Lys-Arg-Cys

at its C-terminal.

Experiment 2-4

Determination of sugar content and structure of sugar chain

This experiment is to reveal the sugar content of the present protein and to the structure of sugar chains as well as to the position of their linkages.

Experiment 2-4(a)

Sugar content

About 5-20μg sugars were detected in 100 μg of the purified protein in Experiment 1 on conventional analysis using mannose as a standard substance. The result shows that the protein contains about 5-20 w/w % sugars, d.s.b.

Experiment 2-4(b)

Sugar component and position of linkage of sugar chain

One mg of the purified protein in Experiment 1 was placed in a reaction tube, admixed with and dissolved to homogeneity in 300 μl of 0.5 M Tris-HCl buffer (pH 8.5) containing 6 M guanidine and 10 mM EDTA, and further admixed with one μl 4-vinylpyridine and 2 μl tri-n-butylphosphine after the tube was filled with nitrogen gas, followed by allowing the resultant mixture to stand for 24 hours under dark conditions to effect pyridylethylation. Thereafter, the reaction mixture was dialyzed against distilled water for 10 hours, and the dialyzed solution was recovered, concentrated, lyophilized, dissolved in 300 μl of 0.2 M ammonium acetate (pH 8.6), admixed with 15 μg trypsin, and incubated at 37 °C for 17 hours.

The resultant trypsin hydrolysates were fed to a column of "218TP54", a column for reverse-phase high-performance liquid chromatography, commercialized by Vydac, California, USA, which had been previously equilibrated with 0.1 v/v % trifluoroacetate in distilled water, and eluted from the column with an acetonitrile solution containing one v/v % trifluoroacetate at a flow rate of 0.5 ml/min while the concentration of acetonitrile was increasing at a rate of one v/v % per minute and the eluate was monitoring at a wave length of 214 nm. From the eluate, fractions containing peptide fragments were recovered, pooled, placed in a test tube, and analyzed for the presence of neutral sugars by the resorcinol-sulfuric acid reaction to reveal that neutral sugars were contained in 2 different types of peptides. The one was named "peptide fragment A", and the other was named "peptide fragment B", and they were analyzed for their amino acid sequences on "MODEL 473A", an amino acid sequencer commercialized by Applied Biosystems, Inc., Foster City, USA.

As a result, the peptide fragments A and B have the following amino acid sequences as shown in Table 1:

Table 1

Peptide fragment A:

Glu-Ala-Phe-Asn-Val-Glu-Asn-Gly-X-Ala-Thr-Pro-Gln-Leu-Thr-Lys

Peptide fragment B:

Thr-Ala-Thr-Asn-Ile-Trp-Ile-Asp-His-Asn-Ser-Phe-Ser-X-Ser-Ser-Asp-Gly-Leu-Val-Asp-Val-Thr-Leu-Thr-Ser-Thr-Gly-Val-Thr-Ile-Ser-Asn-Asn-Leu-Phe-Phe-Asn-His-His-Lys

In order to identify the amino acid residue as indicated by "X" in the amino acid sequences in Table 1, a portion of peptide fragment A or B was placed in a test tube, admixed with an adequate amount of 6 N hydrochloride, and hydrolyzed by incubating it *in vacuo* at 110 °C for 20 hours. After completion of the hydrolysis, the resultant hydrolysates were in usual manner analyzed for their amino acid components on "MODEL 6300", an amino acid analyzer commercialized by Beckman Instruments, Inc., California, USA, to obtain the results as shown in Table 2:

Table 2

Amino acid residue	Amino acid component (number of residue)	
	Peptide fragment	A Peptide fragment B
Asx	2.5(3)	9.0(9)
Thr	1.6(2)	5.5(6)
Ser	0.4(0)	5.9(6)
Glx	2.6(3)	0.0(0)
Pro	0.8(1)	0.0(0)
Gly	1.3(1)	3.5(2)
Ala	1.7(2)	1.3(1)
Cys	0.0(0)	0.0(0)
Val	0.9(1)	2.9(3)
Met	0.0(0)	0.0(0)
Ile	0.0(0)	2.7(2)
Leu	1.1(1)	2.7(3)
Tyr	0.0(0)	0.0(0)
Phe	0.4(1)	2.6(3)
His	0.0(0)	2.8(3)
Lys	1.0(1)	1.4(1)
Arg	0.0(0)	0.0(0)
Note : In the table each numeral in each parenthesis is an approximate integer.		

The comparison of the amino acid sequences as shown in Table 1 and the amino acid components as shown in Table 2 leads to an estimation that the unidentified amino acid "X" in the amino acid sequence of Table 1 might clearly be "Asn" to which a sugar chain is attached.

Experiment 2-4(c)

Isolation of sugar chain and its sugar component

One hundred and fifty mg of the purified protein in Experiment 1 was placed in a container, admixed with and dissolved in a small amount of distilled water, and dialyzed against distilled water for 10 hours. The dialyzed solution was recovered, adjusted to pH 2 by the addition of hydrochloride, admixed with 1.5 mg pepsin, and incubated at 37 °C for 18 hours to cut the peptide bonds in the protein. The reaction mixture was lyophilized, dissolved in 0.1 M citric acid-phosphate buffer (pH 5.5), admixed with 10 m units of "GLYCOPEPTIDASE" commercialized by Seikagaku-Kogyo Co., Ltd., Tokyo, Japan, and incubated at 37 °C for 16 hours to release sugar chains from peptide fragments.

Thereafter, the reaction mixture was fed to a column packed with "BIO-GEL P-4", a gel of Bio-Rad Laboratories Inc., Brussels, Belgium, which had been treated with distilled water to form gel, and the column was then applied with distilled water, followed by collecting eluate by 5 ml fractions and determining the presence of neutral sugars on the resorcinol-sulfuric acid reaction. A portion of a fraction, wherein a neutral sugar had been detected, was placed in a container, lyophilized, dissolved in 60 μl of 4 N hydrochloride containing 0.08 g 2-aminopyridine, incubated at 100 °C for 13 min, admixed with 10 mg sodium cyanotrihydroborate dissolved in 6 μl of distilled water, and further incubated at 90 °C for 18 hours. Finally, the reaction mixture was purified on a column packed with "TSKgel TOYOPEARL® HW-40F", a gel for chromatography commercialized by Tosoh Corporation, Tokyo, Japan, which had been previously equilibrated with 0.01 M ammonium acetate buffer (pH 6.2), to obtain a fluorescent-labelled sugar-chain free of

intact reaction reagents.

The fluorescent-labelled sugar-chain was fed to a column packed with "TSKgel Amido-80", a gel for high-performance liquid chromatography commercialized by Tosoh Corporation, Tokyo, Japan, which had been previously equilibrated with a mixture solution containing 3 v/v % acetic acid-triethylamine buffer (pH 7.3) and acetonitrile in a ratio of 35:65 by volume, and eluted from the column by feeding thereto a liner gradient eluant of 3 v/v % acetic acid-triethylamine buffer and acetonitrile while their volume ratio was increasing to 50:50 in a period of 50 min at a flow rate of one ml/min, followed by the elution of 4 different sugar-chains about 18, 22, 27 and 35 min after the initiation of the elution. The sugar chains were named "sugar-chain **A**", "sugar-chain **B**", "sugar-chain **C**" and "sugar-chain **D**" and subjected to the following analysis for sugar components. The sugar-chains **A** and **C** were detected in a relatively-high level, while the contents of the sugar-chains **B** and **D** were relatively-low.

The sugar-chain **A, B, C** or **D** was lyophilized, admixed with an excessive amount of 4 M trifluoro acetate, and hydrolyzed *in vacuo* at 100 °C for 3 hours. The hydrolysates were dissolved in an adequate amount of pyridine-methanol-distilled water (3:6:2 by volume), admixed with an excessive amount of anhydrous acetic acid, and allowed to stand at ambient temperature for 30 min to effect acetylation. The reaction mixture was recovered, lyophilized, dissolved in 50 μl of 4 N hydrochloride containing 66 mg 2-aminopyridine, heated at 100 °C for 13 min, admixed with 5 μl of distilled water containing 0.8 mg sodium cyanotrihydroborate, and incubated at 90 °C for 18 hours. Thereafter, the reaction mixture was fed to a column packed with "TSKgel G2000PW", a gel for high-performance liquid chromatography commercialized by Tosoh Corporation, Tokyo, Japan, which had been equilibrated with 20 mM ammonium acetate buffer (pH 7.5), and the column was then fed with a fresh preparation of the same buffer at a flow rate of 0.6 ml/min to remove intact reaction reagents. The eluate containing saccharides in mixture was fed to a column packed with "TSKgel Sugar AX-I", a gel for high-performance liquid chromatography, which had been previously equilibrated with 0.5 M borate buffer (pH 8.7) containing 15 v/v % acetonitrile, and the column was fed with a fresh preparation of the same buffer at 60 °C and at a flow rate of 0.4 ml/min, followed by analyzing the types and contents of sugars which consist the sugar chains. The results were as shown in Table 3:

Table 3

Sugar chain	Sugar component				
	Gal	Fuc	Man	Xyl	GlcNAc
Sugar chain A	0.0	1.0	3.0	1.2	3.8
Sugar chain B	1.0	1.0	3.0	1.1	3.5
Sugar chain C	1.1	2.1	3.0	0.8	4.1
Sugar chain D	2.1	3.1	3.0	0.8	3.8
Note : In the table each numeral represents a relative value provided the value of Man, i.e. mannose, is 3.0.					

The results in Table 3 indicate that the sugar chains **A** to **D** attached to the present protein consist of 0 to 2 galactose residues (Gal), 1 to 3 fucose residues (Fuc), 3 mannose residues (Man), one xylose residue (Xyl) and 4 N-acetylglucosamine residues (GlcNAc), and at least one of these sugar chains are attached to the present protein.

Experiment 2-4(d)

Determination of sugar-chain structure

The sugar chains **A** to **D**, isolated in Experiment 2-4(c), were respectively placed in containers, lyophilized, dissolved in a small amount of heavy water, and lyophilized. The above procedure was repeated thrice for each sugar chain to replace dissociative hydrogens in each sugar chain with deuterium. Thereafter, the resultant was analyzed on a 500-MHz ^{1}H NMR (nuclear magnetic resonance) spectroscope, commercialized by JEOL U.S.A., Inc., Peabody, MA, USA, to analyze the chemical shifts of anomeric- and methyl-protons in each sugar chain at 30 °C in heavy water. The results are as shown in Table 4.

The present inventors investigated conventionally known sugar chains which exhibit similar chemical shifts to those of the aforesaid sugar chains of **A** to **D**, and found that, as shown in *Biochemistry,* Vol.25, pp.388-395 (1986) reported by N. TAKAHASHI et al., the sugar chains **c, e** and **f** attaching to laccase, i.e. an enzyme derived from sycamore (*Acer pseudoplatanus* L.), exhibits nearly the same chemical shifts as those exhibited by the sugar chains **A** to **D** when assayed on 400-MHz ^{1}H NMR. The chemical shifts of the anomeric- and methyl-protons in the sugar chains **c, e** and **f** attached to laccase are also shown in Table 4:

Table 4

Sugar chain	Chemical shift of anomeric proton (δppm, D_2O)										
	GlcNAc-2	Man-3	Man-4	Man-4'	GlcNAc-5	GlcNAc-5'	Gal-6	Gal-6'	Fuc-1	Fuc-2	Xyl
c	4.592	4.857	5.140	4.899	4.511	4.536	-	-	5.048	-	4.425
A	4.596	4.849	5.139	4.897	4.512	4.544	-	-	5.042	-	4.427
B	4.630	4.860	5.137	4.898	4.513	4.579	-	4.503	-	5.005	4.415
e	4.591	4.855	5.139	4.900	4.511	4.576	-	4.499	5.047	5.000	4.424
C	4.592	4.849	5.139	4.901	4.514	4.578	-	4.499	5.042	5.001	4.427
f	4.592	4.856	5.139	4.901	4.533	4.576	4.490	4.499	5.048	4.499	4.423
D	4.593	4.853	5.139	4.900	4.534	4.578	4.491	4.498	5.045	5.001	4.424

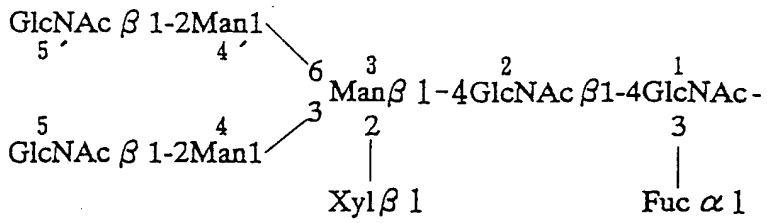
Sugar chain	Chemical shift of methyl proton (δppm, D_2O)				
	GlcNAc-2	GlcNAc-5	GlcNAc-5'	Fuc-1	Fuc-2
c	2.043	2.043	2.043	1.197	-
A	2.044	2.044	2.044	1.192	-
B	2.041	2.041	2.041	-	-*
e	2.042	2.042	2.042	1.195	1.170
C	2.043	2.043	2.043	1.191	1.171
f	2.042	2.042	2.042	1.197	1.170
D	2.041	2.041	2.041	1.195	1.170

Note: Chemical shifts were measured with internal acetone (δ=2.216 ppm) at 30°C. In the table, the symbol "Fuc-1" represents fucose residue which couples to GlcNAc-1; the symbol "Fuc-2" represents fucose residue which couples to GlcNAc-2 or GlcNAc-5', and the symbols "Gal-6" and "Gal-6'" represent galactose residue which couples to GlcNAc-5 or GlcNAc-5'. The symbol "-*" represents that no signal was detectable due to the limited amount of the sample.

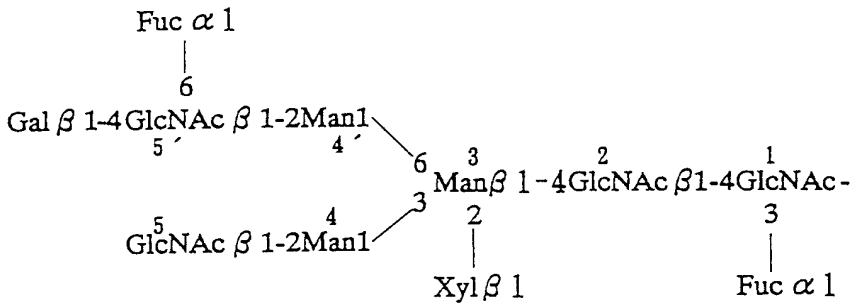
As shown in Table 4, the sugar chains **c, e** and **f,** which were reported by N. TAKAHASHI et al., and shown by the following chemical formulae 2, 3, and 4, have N-linked sugar-chain structures consisting of 4 N-acetylglucosamine residues and 3 mannose residues having side chains of Fucα1, Xylβ1 and/or Galβ1. Based on these findings, the sugar chains **A** to **D** are respectively estimated to have the following structures as shown by the chemical formulae 5, 6, 7 and 8, and, therefore, the sugar chains which are attached to the

present protein can be represented by the general formula 2. Now briefly supplements the nuclear magnetic resonance spectrum of the sugar chain **B,** no signal of anomeric- and methyl-protons corresponding to that of Fuc-1 residue in Table 4 was detectable, and the chemical-shift value of GlcNAc-2 was shifted to a low magnetic field of 4.630 ppm which is lower than 4.591 ppm as in the sugar chain **e**. The facts represent that the sugar chain **B** has a structure of the sugar chain **e** wherein Fucα1 is lacking.

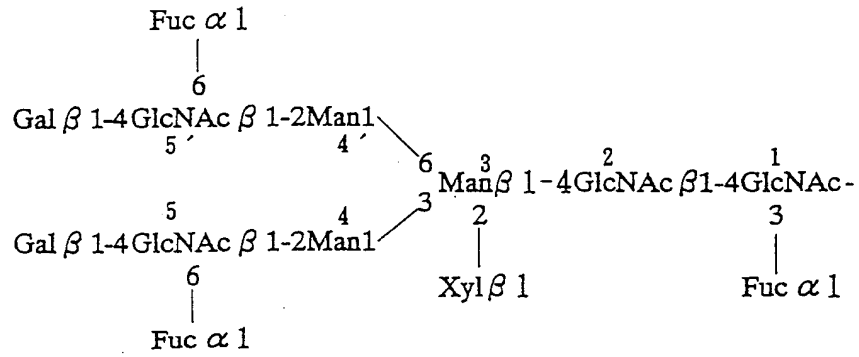
Chemical formula 2:
(Sugar chain of laccase c)



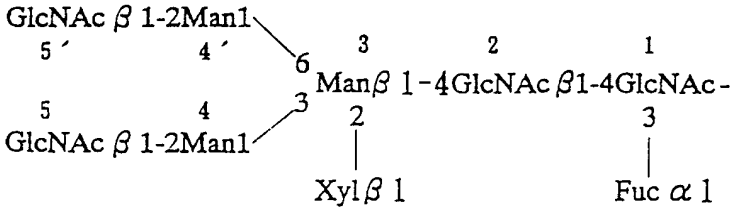
Chemical formula 3:
(Sugar chain of laccase e)



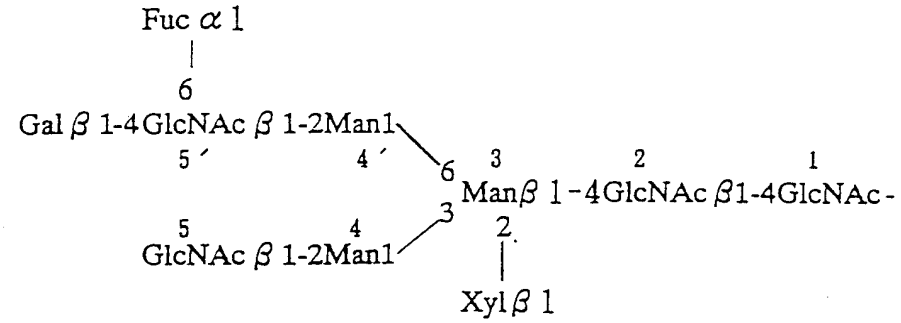
Chemical formula 4:
(Sugar chain of laccase f)



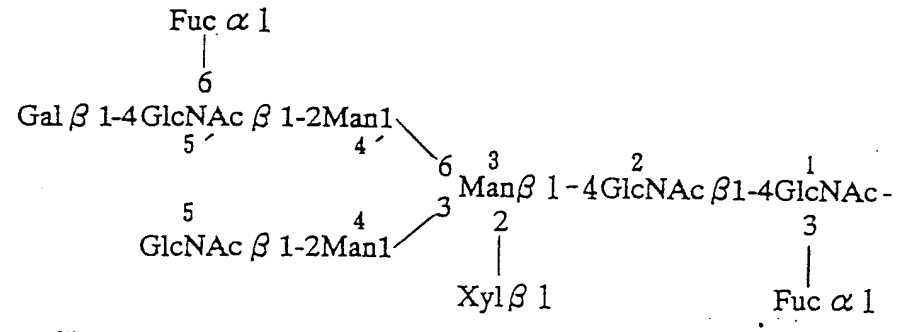
Chemical formula 5:
(Sugar chain A)



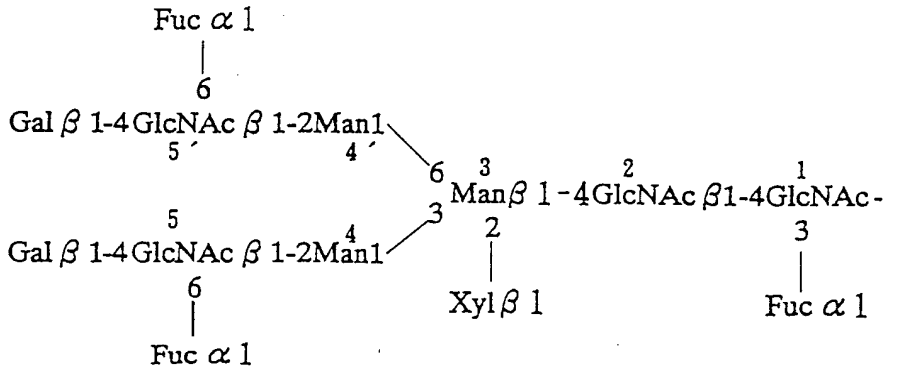
Chemical formula 6:
(Sugar chain B)



Chemical formula 7:
(Sugar chain C)



Chemical formula 8:
(Sugar chain D)



Experiment 2-5

Ultraviolet absorption spectrum

The purified protein obtained in Experiment 1 exhibited the maximum ultraviolet absorption spectrum at a wave length around 280 nm when measured with a spectrophotometer in an aqueous solution.

Experiment 2-6

Solubility in solvent

The purified protein obtained in Experiment 1 was soluble in water, physiological saline and phosphate buffer when tested in usual manner.

Experiment 2-7

Biological activity

The purified protein obtained in Experiment 1 has features that it induces the growth of T-cells which specifically react with the protein, and couples to an immunoglobulin E antibody prepared from a patient with pollenosis when tested by the following experiments. The sugar chain attached to the present protein has a specific immunological property, and the removal thereof from the protein results in a significant reduction of the antigenicity of the protein.

Experiment 2-7(a)

Test on T-cell growth induction

By using ficoll-hypaque gradient centrifugation, mononuclear cells were isolated from a heparinized peripheral blood which had been collected from a patient with pollenosis. The mononuclear cells were suspended in RPMI 1640 medium (pH 7.0) supplemented with 10 v/v % AB serum to give a cell density of $1x10^6$ cells/ml, admixed with 20 μg/ml of the purified protein in Experiment 1, and intubated in an incubator at 37 °C for 5 days in 5 v/v % CO_2 atmospheric conditions. Thereafter, 50 units/ml of a recombinant human interleukin 2 was added to the resultant culture which was then further incubated similarly as above for 9 days. The resultant cells, pretreated in this manner, were used in the following T-cell growth test.

To 96-well microplate were added $4x10^4$ cells/well of the mononuclear cells suspended in RPMI 1640 medium (pH 7.0) supplemented with 10 v/v % AB serum, $1x10^6$ cells/well of peripheral mononuclear cells which had been collected from the same patient and incubated at 37 °C for 30 min in the presence of 50 μg/ml mitomycin C, 50 μg/ml of the purified protein in Experiment 1, and a fresh preparation of the same medium as used above to give a total volume of 200 μl/well. The cells were successively incubated under 5 v/v % CO_2 atmospheric conditions at 37 °C for 2 days, admixed with 0.5 μCi/well ^{3}H-thymidine, and incubated for one day, followed by counting the uptake amount of ^{3}H-thymidine on a scintillation counter. In parallel, a system using a medium free of the present protein was arranged as control and treated similarly as above. As a result, the control system showed about 200 cpm uptake of ^{3}H-thymidine, while the system with the protein showed about 8,500 cpm uptake of ^{3}H-thymidine per 50 μg/ml of the purified protein, and this revealed that the purified protein might strongly accelerate the T-cell growth in pollenosis patients' blood. This also means that the protein has an antigenicity.

Experiment 2-7(b)

Test on conjugation with immunoglobulin E antibody

Two hundred and fifty μg of the purified protein in Experiment 1 was dissolved in a small amount of distilled water, and the resultant solution was dialyzed against distilled water for 18 hours. The dialyzed solution was lyophilized, dissolved in 125 μl of 2 M acetate buffer (pH 7.8) containing 0.5 w/v % sodium dodecyl sulfate (SDS) and 50 mM 2-mercaptoethanol, and heated at about 100 °C for 10 min. The resultant solution was admixed with 60 μl of 7.5 w/v % nonidet P-40, a nonionic detergent, 5 units of N-glycanase, 200 μl of distilled water, and incubated at 37 °C for 24 hours to release sugar chains from the protein. As a control, a system, wherein N-glycanase was not used, was schemed and treated similarly as in the above.

To 96-well microplate was adsorbed the purified protein in Experiment 1 in an amount of one μg/well, and a serum preparation prepared from a healthy volunteer or a patient with pollenosis was added to the wells in an amount of 100 μl/well, followed by incubating the microplate at 37 °C for 2 hours. The microplate was washed with 0.1 M phosphate buffer (pH 7.2) containing 0.1 v/v % calf serum albumin to remove non-adsorbed serum, and an anti-human immunoglobulin E antibody, prepared with goat and labelled with peroxidase from horseradish, was added to the wells of the microplate in an amount of 100

μl/well, followed by further incubating the microplate at 37 °C for 2 hours. Thereafter, the microplate was washed with a fresh preparation of the same phosphate buffer used above to remove non-adsorbed antibody, and to each well of which was added 100 μl of 0.1 M citrate buffer (pH 5.0) containing 0.5 mg/l of o-phenylenediamine and 0.03 v/v % hydrogen peroxidase to effect coloration, followed by measuring the absorbance of a solution in each well at 492 nm.

As a result, the absorbance in the system using the protein free of sugar chain was about 0.05, while that in the system using the protein with sugar chain(s) was as high as about 0.3, and this indicates that the present protein specifically conjugates with immunoglobulin E antibodies contained in blood of patients with pollenosis, and that the sugar chains are inevitable to exhibit the antigenicity of the protein. Furthermore, the results in Experiment 2-7 confirm the fact that the present protein is a substance causative of pollenosis, i.e., it has a property to induce pollenosis in mammals including human.

Experiment 2-8

Stability

No residual activity was detected when the purified protein in Experiment 1 was incubated in an aqueous solution (pH 7.2) at 100 °C for 10 min. No substantial loss of activity was observed even after a 1-month incubation of the purified protein in an aqueous solution (pH 7.2) at 4 °C.

Any protein having these properties has not yet been known, and this concludes that the present protein is a novel substance.

Now explaining the process to produce the present protein, it can be prepared by collecting pollen from cedar such as "*omote−sugi*" or "*ura−sugi*", i.e. *Cryptomeria japonica,* extracting the pollen with an aqueous solvent and purifying the extract. The methods to extract the present protein from cedar pollens used in the invention are generally those which comprise collecting pollen from male flowers of cedar; suspending the pollen in water or an aqueous solvent such as those of a readily water-soluble organic solvent of methyl alcohol, ethyl alcohol, acetone or the like, or in an aqueous solvent admixed with an adequate amount of a stabilizer or the like; and soaking the pollen while stirring if needs arise in such a solvent at a temperature lower than 10 °C, preferably, about 0-5 °C, for 30 min or longer, preferably, about 1-2 hours. Depending on the conditions of a cedar pollen used, the aforesaid procedure is usually carried out 1 to 5 times to extract the most of the protein from the material cedar pollen.

The protein in the resultant extract can be purified by conventional techniques in general used in this field. Partially-purified protein can be obtained from the extract by using a method such as a salting out, dialysis, filtration, centrifugation, gel filtration chromatography, etc. Such a partially-purified protein generally contains cedar pollen allergens such as *Cry j* II together with the present protein. In case of a more highly-purified protein is required, it is obtainable by removing components such as *Cry j* II and contaminants other than the present protein with one or more methods such as a gel filtration chromatography, ion-exchange chromatography, affinity chromatography, electrophoresis, isoelectrophoresis, etc.

The partially purified- and highly purified- proteins thus obtained can be arbitrarily incorporated into or used as desensitization agent for diagnosing, treating and/or preventing pollenosis. The purified protein is useful as antigen for detecting immunoglobulin E antibodies, which have a specificity to the protein, for qualitative and quantitative analyses on enzyme immunoassay and radioimmunoassay, and extensively applicable in the diagnosis of pollenosis and the scientific study to reveal the induction mechanisms of allergies in general.

The following examples concretely explain the process to produce the present protein:

Example A-1

Preparation of partially-purified protein

One part by weight of a cedar pollen prepared from male flowers of "*omote−sugi*", i.e. *Cryptomeria japonica*, grown in Chiba, Japan, was suspended in about 16 parts by weight of an aqueous solution of 0.125 M sodium hydrogen carbonate (pH 8.2) to effect extraction while stirring at 4 °C for one hour, followed by centrifuging the resultant extract to obtain a supernatant. The sediment was treated similarly as above to obtain a supernatant which was then pooled with the above supernatant. To the mixture was added 0.1 w/v % "CETAVLON", hexadecyltrimethylammonium bromide commercialized by Sigma, Chemicals Co., Louis, USA, and the mixture was centrifuged to obtain a supernatant which was then mixed with ammonium sulfate to give a saturation degree of 80 w/w % to salt out proteinaceous components. The resultant sediment was

dialyzed against 50 mM Tris-HCl buffer (pH 7.8) for 10 hours, and the dialyzed solution was filtered to obtain a filtrate which was then fed to a column packed with DEAE-SEPHADEX® which had been previously equilibrated with 50 mM Tris-HCl buffer (pH 7.8), followed by recovering non-adsorbed fractions. The non-adsorbed fractions were pooled, adjusted to pH 5.0 by the addition of acetic acid, and fed to a column packed with "CM-SEPHADEX" which had been previously equilibrated with 10 mM acetate buffer (pH 5.0). The column was washed by feeding thereto 10 mM acetate buffer (pH 5.0), and fed with an eluant consisting of 0.1 M phosphate buffer (pH 7.0) and 0.3 M sodium chloride to elute the proteinaceous components. Thereafter, the resultant eluate was concentrated and lyophilized to obtain a partially-purified protein containing *Cry j* II along with the present protein. The yield was about 0.1% against the weight of the material cedar pollen, d.s.b.

The partially purified protein thus obtained can be suitably used as desensitization agent for diagnosing, treating and/or preventing pollenosis.

Example A-2

Purified protein

A partially-purified protein obtained by the method in Example A-1 was dissolved in a small amount of distilled water, and the solution was fed to a column packed with "MONO-S" which had been previously equilibrated with 10 mM phosphate buffer (pH 5.0). The column was fed with a liner gradient buffer of salt increasing from 0 M to 0.5 M in 10 mM Tris-HCl buffer (pH 7.0), followed by the elution of the present protein at a salt concentration of 0.1 M to 0.3 M. Thereafter, the eluate was concentrated and lyophilized to obtain a purified protein substantially consisting of the present protein. The yield was about 0.02% against the weight of the material cedar pollen, d.s.b.

The purified protein can be suitably used as desensitization agent for diagnosing, treating and/or preventing pollenosis, as well as being used as antigen for enzyme immunoassay and radioimmunoassay.

The uses of the present protein will be explained with reference to the following examples and experiments.

Since the present protein is one of the major substances causative of pollenosis, it can be extensively used as desensitization agent for diagnosing, treating and/or preventing pollenosis. The desensitization agent according to the present invention comprises as an effective ingredient the present protein or the later described conjugate of the protein and a specific saccharide. As regards a desensitization agent directed to diagnosis of pollenosis, it can be generally prepared by mixing with a carrier a partially purified- or a highly purified-protein prepared by the aforesaid methods. As regards a desensitization agent directed to the treatment and/or prevention of pollenosis, it can be prepared into a conjugate prior to the mixing as mentioned above by allowing the present protein to couple up to a specific saccharide.

The specific saccharides as referred to in the invention include those which can covalently couple to the present protein to form conjugates whereby the desensitization efficacy of the protein is significantly augmented and/or the side effects are significantly reduced. Examples of such a saccharide are homo or heteropolysaccharides such as starch, amylose, dextran, polysucrose, pullulan, elsinan, curdlan, gum arabic, gum tragacanth, guar gum, xanthan gum, carrageenan, cellulose, glucomannan, chitosan, lipopolysaccharides, and their derivatives and partial hydrolysates. Usually, the average molecular weight of such a saccharide is in the range of about 500-10,000,000 daltons, preferably, about 10,000-1,000,000 daltons. Among these saccharides, pullulan and elsinan, which substantially consist of repeating maltotriose units, as well as their partial hydrolysates, are prepared with the protein into conjugates which have an activity of inducing immunoglobulin G and M antibodies which are effective for desensitization by a large margin when administered to mammals including human, but do not substantially form immunoglobulin E antibody, a major factor causative of unsatisfactory side effects including anaphylactic shock. These properties are advantageously useful in the desensitization therapy which requires a repeated administration of medicaments in order to attain a considerably-high effect without a fear of causing unsatisfactory side effects.

For example, lipopolysaccharides derived from microorganisms of *Escherichia coli, Salmonella, Serratia,* etc. and their partial hydrolysates exert the following features when prepared with the present protein into conjugates: Such a lipopolysaccharide increases the affinity of the protein against the mucosae of mammals and significantly improves the intake efficiency of the protein. For this reason, conjugates of the present protein and the saccharides are advantageously useful as desensitization agents which are premised on percutaneous and/or permucosal administration.

Usually, such a conjugate can be prepared by reacting the present protein with an activated saccharide or by bridging the protein with a saccharide by using a reagent having one or more active functional groups. Examples of such a reaction are the diazo method, peptide method, alkylation method, bridging method, amide binding method, peroxidase oxidation method, disulfide binding method, etc., and these methods per se are known in this art. A representative of such a method is described in detail, for example, in Japanese Patent Laid-Open No.93,730/91. The ratio of the protein against a saccharide at the initiation of the reaction suitably used in the invention is usually in the range of about 1:0.001 to 1:1,000, preferably, about 1:0.01 to 1:100, d.s.b. Depending on the reaction methods used, molecules of the protein readily couple each other when the ratio is below the range, while molecules of the saccharide are readily react each other when the ratio exceeds the range. Anyway, any ratios other than those specified in the invention result in reductions of reactivity and purification efficiency of the reaction products, and this concluded that the above-specified ratios are the best mode. The reaction temperature, pH and time used in the present invention are chosen so as not to inactivate or decompose the protein or to reduce the side reactions as much as possible, and, usually, a temperature of about 0-100 °C and a pH of about 0.1-12 are suitably used to complete the reaction within about 0.1-50 hours.

The conjugates obtained by the above reaction can be purified by conventional methods in general such as dialysis, salting out, filtration, concentration, centrifugation, gel filtration chromatography, ion-exchange chromatography, affinity chromatography, electrophoresis, isoelectrophoresis, etc., and, if necessary, such a conjugate can be purified by an appropriate combination use of the above methods. The resultant purified conjugates may be concentrated and lyophilized into liquid or solid products to meet to final use.

The desensitization agent according to the present invention includes the aforesaid protein and/or conjugates, and other compositions comprising either of the protein or a conjugate along with a physiologically acceptable carrier, excipient, diluent, adjuvant and/or stabilizer, and other one or more medicaments, for example, an antihistaminic and an antiinflammatory agent such as steroid hormone or disodium cromoglycate. The desensitization agent may be in a dose form, i.e. those which contain the present protein or conjugate in an amount suitable for a dose per day or in an amount up to 4 times by integers or up to 1/40 times of the dose, and may be a physically separated form suitable for a prescribed administration. Examples of the form of such a pharmaceutical agent are a powder, parvule, granule, pearl, tablet, capsule, troche, syrup, emulsion, ointment, emplastrum, pap, suppository, collyrium, collunarium, nebula, injection, etc.

Now explaining the use of the present desensitization agent, it can be used similarly as conventional desensitization agents in general containing cedar pollen allergens. In case of using the present desensitization agent for diagnosing pollenosis, patients are scratched on their skin surfaces with a care not to bleed by using conventional tests known as the scratch or intradermal-test, and an adequate amount of the present desensitization agent for diagnosis was dropped on the scratched sites, or they are intradermally injected with an adequate amount of the desensitization agent. Thereafter, the occurrence and the size of urtica formed 15-30 min after the dropping or injecting are examined and measured, and it was determined to be positive when the size exceeded a prescribed level.

In the treatment using the present desensitization agent, an appropriate dose and application thereof are usually determined based on the results of the aforesaid diagnosis. Patients with a positive result in the diagnosis are orally or parenterally administered with the present desensitization agent containing a conjugate of the present protein and a specific saccharide. Depending on the symptoms and/or administration routes, patients are usually administered repeatedly via the route of an intradermal, subcutaneous, intramuscular, intraperitoneal or permucosal administration with the present desensitization agent at a dose of about 0.0001-100,000 ng/shot/adult, preferably, a dose of about 0.001-10,000 ng/shot/adult and a frequency of one shot per week or month for about 1-12 months while usually increasing the dose. In case of using the present desensitization agent in the prevention of pollenosis, approximately the same dose and application as used in the treatment of pollenosis can be used, and patients are usually administered repeatedly with the desensitization agent at a dose of about 0.0001-100,000 ng/shot/adult, preferably, a dose of abut 0.001-10,000 ng/shot/adult, and a frequency of one shot per week or month for about 1-6 months while usually increasing the dose via the route of an intradermal, subcutaneous, intramuscular or permucosal administration while observing the patients' conditions and symptoms. When the present desensitization agent is repeatedly administered to patients in a prescribed time interval from the beginning of autumn to the following early spring, predictable allergic symptoms of the patients which might be induced in the forthcoming season would be substantially reduced or completely avoided.

The desensitization agent according to the present invention will be described concretely with reference to the following several examples:

Example B-1

Dried injection

Two g of a purified pullulan having an average molecular weight of about 200,000 daltons was dissolved in 100 ml distilled water, and the solution was mixed with 2 ml of 1.7 w/v % cyanuric chloride in acetone solution. Thereafter, the resultant mixture was reacted by allowing it to stand at 4 °C or lower for 2 hours in an ice-chilled water bath while the pH was controlled to around 7 by the addition of 5 w/v % aqueous sodium carbonate solution. The resultant solution containing an activated pullulan was mixed with 40 mg of a purified protein obtained by the method in Example A-2, and allowed to react at 37 °C and pH 7.0 for 5 hours under stirring conditions. After completion of the reaction the resultant was mixed with one w/v % glycine and incubated at 37 °C for 2 hour while stirring to block the intact activated groups, and the resultant mixture was dialyzed against 0.01 M acetate buffer (pH 5.0) for 5 hours, fed to a column packed with "CM-SEPHADEX" which had been previously equilibrated with 0.01 M acetate buffer (pH 5.0), followed by recovering the resultant conjugate of the protein and pullulan from non-adsorbed fractions. The conjugate was in usual manner dissolved in physiological saline supplemented with one w/v % human serum albumin to give a final concentration of about 100 ng/ml, and the solution was membrane filtered, distributed to sterile vials by 2 ml aliquots, lyophilized and cap sealed.

In use, one ml distilled water for injection is added to each vial, and the contents are dissolved to homogeneity prior to administration. The product, which contains the conjugate of the protein and pullulan as effective ingredient, can be arbitrarily useful as dried injection for treating and/or preventing pollenosis.

Example B-2

Injection

One g of CM-cellulose having an average molecular weight of about 20,000 daltons was dissolved in 200 ml distilled water, and the solution was mixed with 2 g 1-ethyl-3-(3-dimethyl-aminopropyl)carbodiimide-methiozide, followed by reacting the resultant solution at ambient temperature for 2 hours while stirring and keeping at a pH of around 4 by the addition of 1 N hydrochloride. The reaction mixture was dialyzed against distilled water for 24 hours, and the dialyzed solution was recovered, mixed with 30 mg of a purified protein obtained by the method in Example A-2, and allowed to stand to react the contents at ambient temperature and pH 4.5 for 15 hours. Thereafter, the resultant conjugate in the reaction mixture was purified similarly as in Example B-1, concentrated, dissolved in 50 v/v % aqueous glycerine solution, membrane filtered, distributed to sterile vials by 2 ml aliquots, and cap sealed.

With reference to the diagnostic results of the scratch-or intradermal-test, the product is admixed with 100-fold to 100,000-fold volumes of 50 v/v % aqueous glycerine solution to dilute the contents to homogeneity prior to administration. The product containing the conjugate of the present protein and CM-cellulose can be arbitrarily used as desensitization agent for treating and/or preventing pollenosis.

Example B-3

Liquid preparation

One hundred mg of a purified lipopolysaccharide derived from a microorganism of the genus *Salmonella* in 25 ml of an about 4 °C aqueous solution of sodium acetate with a saturation degree of 50 w/v %, and the resultant solution was adjusted to pH 9.0 by the addition of 0.5 N sodium hydroxide, followed by adding thereto drop by drop one ml anhydrous dioxane containing 20 μl bromoacetyl bromide while keeping at a pH around 8.5. Thereafter, the resultant solution was adjusted to a pH of about 4.5 by the addition of 6 N acetic acid, dialyzed against 4 °C distilled water for 48 hours to obtain an aqueous solution containing an activated lipopolysaccharide. To the aqueous solution was added 40 mg of a purified protein obtained by the method in Example A-1, followed by allowing the mixture to react at ambient temperature for 48 hours while keeping at a pH of about 4.5. Thereafter, the reaction mixture was purified similarly as in Example B-1, concentrated, lyophilized to obtain a solid conjugate of the present protein and lipopolysaccharide. The solid conjugate was dissolved in distilled water containing one w/v % purified gelatin to give a final concentration of 100 ng/ml, and the resultant solution was in usual manner membrane filtered to obtain a liquid preparation.

The product, containing a conjugate of the present protein and lipopolysaccharide, can be arbitrarily used as liquid preparation for a collyrium, collunarium or nebula for oral cavity to treat and/or prevent pollenosis.

Example B-4

Sublingual tablet

A purified elsinan having an average molecular weight of about 200,000 daltons was dissolved to homogeneity in 400 ml distilled water, and the solution was adjusted to pH 10.7 by the addition of 1 N sodium hydroxide, gradually admixed with 3 g cyanogen bromide while keeping at a pH around 10.0, and reacted for one hour. The reaction mixture was adjusted to pH 5.0 by the addition of 1 N hydrochloride, and dialyzed against cold water for 10 hours while keeping at the pH, followed by recovering an aqueous solution containing an activated elsinan. To the aqueous solution was added 20 mg of a purified protein obtained by the method in Example A-2, and the resultant was allowed to stand at ambient temperature for 24 hours to react the contents. After completion of the reaction, the reaction mixture was mixed with 3-fold volumes of acetone, followed by recovering the formed precipitate, dissolving it in 0.01 M acetate buffer (pH 5.0), and removing insoluble substances by centrifugation. The supernatant thus obtained was fed to a column packed with "CM-SEPHADEX" which had been previously equilibrated with 0.01 M acetate buffer (pH 5.0), followed by recovering fractions containing a conjugate of the present protein and elsinan. The fractions were in usual manner pooled, membrane filtered, concentrated, pulverized and mixed to homogeneity with "FINETOSE®", an anhydrous crystalline α-maltose powder commercialized by Hayashibara Co., Ltd., Okayama, Japan. The resultant mixture was in usual manner tabletted with a tabletting machine to obtain tablets, 200 mg weight each, containing 100 ng of the present protein per tablet.

The product, having a satisfactory stability and applicability, can be arbitrarily used as sublingual agent for treating and/or preventing pollenosis.

Example B-5

Diagnostic agent

Ten mg of a partially-purified protein obtained by the method in Example A-1 was dissolved in 20 ml of physiological saline, and the solution was in usual manner membrane filtered, distributed to sterile vials by one ml aliquots, lyophilized and cap sealed.

The product is dissolved in one ml of distilled water for injection, diluted with 10 folds with a fresh preparation of the same distilled water prior to use, and used in the diagnosis of pollenosis by the scratch- and intradermal-tests.

Example B-6

Diagnostic agent

One mg of a purified protein obtained by the method in Example A-2 was dissolved in 20 ml of 50 v/v % glycerine containing 5 w/v % sodium chloride, and the resultant solution was in usual manner membrane filtered and distributed to sterile vials by one ml aliquots.

The product is diluted by 20-fold with a 50 v/v % aqueous glycerine solution and used in the diagnosis of pollenosis by the scratch- and intradermal-tests.

The following several experiments explain the efficacy of the present desensitization agent:

Experiment 3

Animal experiment

This experiment was carried out to evidently show the fact that the present conjugate of the protein and a specific saccharide according to the invention exerts an efficacy on the treatment and/or prevention of pollenosis when administered to experimental animals.

Experiment 3-1

Prophylactic effect

Six female BALB/c mice, 10-12-week-old, in a group, were intraperitoneally injected with a desensitization agent obtained by the method in Example B-1 in a dose of one μg/mouse of the protein per week over 3 weeks. One week after the final injection, the mice were induced pollenosis by injecting to them similarly as above 0.2 ml of a physiological saline, containing 4 mg aluminum hydroxide as adjuvant and one μg of a purified protein obtained by the method in Example A-2 as antigen. The mice were sampled their blood immediately before and one week after the administration of the antigen, and the blood samples were examined for the amounts of immunoglobulin G and M antibodies specific to the protein.

As a control system, mice were administered with a mixture containing a purified protein, obtained by the method in Example A-2, and a purified pullulan having an average molecular weight of about 200,000 daltons in a weight ratio of 1:15, and treated similarly as above. The amount of immunoglobulin E antibody which is specific to the present protein was assayed by the passive cutaneous anaphylaxis (PCA) reaction as reported by I. Mota and D. Wong in *Life Sciences,* Vol.8, No.16, Part II, pp.813-820 (1969), and the amount of immunoglobulin M antibody which is specific to the present protein was assayed by the enzyme immunoassay as reported by S. YOSHITAKE in *The Journal of Biochemistry,* Vol.92, No.5, pp.1,413-1,424 (1982). The amount of each antibody was expressed with an average value of antibody titers of 6 mice. The results were as shown in Table 1:

Table 5

Desensitization agent	Immediately before administration		One week after administration		Judgement
	IgG + IgM	IgE	IgG+ IgM	IgE	
Conjugate of protein and pullulan	235	0	970	3	Present invention
Mixture of protein and pullulan	30	15	245	320	Control

Note : In the table, "protein" means the present protein; "IgG", immunoglobulin G; "IgM", immunoglobulin M; and "IgE", immunoglobulin E.

As is evident from the results in Table 5, compared with the control system, the system, wherein the mice had been previously administered with the desensitization agent containing the conjugate of the present protein and pullulan, showed a relatively-high productivity of immunoglobulin G and M antibodies which are effective for desensitization, while the formation of immunoglobulin E antibody in the mice was substantially inhibited. The inhibitory activity of forming immunoglobulin E antibody, exerted by the present

desensitization agent by administering it to mice, indicates that the agent can be safely and effectively used in the prevention of pollenosis of mammals including human with the viewpoint of that immunoglobulin E antibody is known as a major factor causative of unsatisfactory side effects including anaphylactic shock.

Experiment 3-2

Therapeutic effect by parenteral administration

Pollenosis was induced in 6 female BALB/c mice, 10-12-week-old, in a group, by intraperitoneally injecting them once a week over 3 weeks with 0.2 ml of a physiological saline containing one μg of a purified protein as antigen, obtained by the method in Example A-2, and 4 mg aluminum hydroxide as adjuvant. One week after the final administration of the antigen, the mice were injected once a week over 3 weeks similarly as above with a desensitization agent obtained by the method in Example B-1 at a dose of 100 ng/mouse of the protein, d.s.b. One week after the final administration of the desensitization agent, the mice were further administered only with the antigen to reinduce immunoglobulin E antibody. The mice were sampled their blood immediately before the administration of the desensitization agent, one week after the final administration of the desensitization agent, and one week after the reinduction of immunoglobulin E antibody, and the blood samples were assayed by the same method as in Experiment 3-1 for determining the amounts of immunoglobulin E, G and M antibodies which are specific to the protein.

As a control system, mice were administered with a mixture, containing a purified protein, obtained by the method in Example A-2, and a purified pullulan having an average molecular weight of about 200,000 daltons in a weight ratio of 1:15 in place of the desensitization agent as used above, and treated similarly as above. The results were as shown in Table 6:

Table 6

Desensitization agent	Immediately before administration		One week after administration		One week after reinduction of IgE		Judgement
	IgG + IgM	IgE	IgG + IgM	IgE	IgG + IgM	IgE	
Conjugate of protein and pullulan	350	185	2,550	35	5,800	45	Present invention
Mixture of protein and pullulan	340	180	475	370	2,400	1,300	Control

Note : In the table, "protein" means the present protein; "IgG", immunoglobulin G; "IgM", immunoglobulin M; and "IgE", immunoglobulin E.

As is evident from the results in Table 6, compared with the control system, the system, wherein the mice were administered with the present desensitization agent containing a conjugate of the present protein and pullulan, resulted in the formation of relatively-large amounts of immunoglobulin G and M antibodies both after the administration of the desensitization agent and the reinduction of immunoglobulin E antibody. As regards immunoglobulin E antibody, the formation was substantially inhibited even before the administra-

tion of the desensitization agent and after the reinduction of immunoglobulin E antibody. These results confirm that pollenosis of mammals including human could be safely and effectively treated by the parenteral administration of the present desensitization agent containing the conjugate.

Experiment 3-3

Therapeutic effect by oral administration

Pollenosis was induced in 6 female BALB/c mice, 10-12-week-old, in a group, by intraperitoneally injecting to them once a week over 3 weeks 0.2 ml of a physiological saline containing one μg of a purified protein as antigen, obtained by the method in Example A-2, and 4 mg aluminum hydroxide as adjuvant. One week after the final administration of the antigen, the mice were orally administered once a week over 3 weeks similarly as above with a sublingual agent, obtained by the method in Example B-4, at a dose of 100 ng/mouse of the protein, d.s.b. One week after the final administration of the sublingual agent, the mice were sampled their blood, and the blood samples were assayed for the amounts of immunoglobulin A, G and E antibodies which are specific to the protein.

As a control system, mice were orally administered with a solid mixture containing a purified protein, obtained by the method in Example A-2, and a purified lipopolysaccharide derived from *Salmonella* in a weight ratio of 1:15, and treated similarly as above. The amounts of immunoglobulin A and G antibodies, which are specific to the present protein, were assayed with the enzyme immunoassay (EIA) as reported by R. Maiolini et al. in *Journal of Immunological Methods*, Vol.6, pp.355-362 (1975), and the amount of immunoglobulin E antibody was assayed with the same method as in Experiment 3-1. The amounts of immunoglobulin A, G and E antibodies were respectively expressed with an average value of antibody titers of 6 mice. The results were as shown in Table 7:

Table 7

Desensitization agent	Immediately before administration		One week after administration		Judgement
	IgA + IgG	IgE	IgA + IgG	IgE	
Conjugate of protein and lipopolysaccharide	330	165	2,250	25	Present invention
Mixture of protein and lipopolysaccharide	325	185	410	310	Control

Note : In the table, "protein" means the present protein; "IgA", immunoglobulin A; "IgG", immunoglobulin G; and "IgE", immunoglobulin E.

As is evident from the results in Table 7, compared with the control system, the system, wherein the mice were administered with the desensitization agent containing the conjugate of the present protein and lipopolysaccharide, showed a relatively-large productivity of immunoglobulin A and G antibodies, while the production of immunoglobulin E was substantially inhibited. These results confirm that the present desensitization agent containing the conjugate can safely and effectively treat pollenosis of mammals

including human even when administered orally.

Although the data are not shown, significant therapeutic and/or prophylactic effects were exerted on pollenosis without a fear of causing unsatisfactory side effects even when mice, rats and guinea pigs were intradermally, subcutaneously, intramuscularly or intraperitoneally administered by conventional methods as used in this field with a desensitization agent obtained by the methods in Examples B-1 to B-4, or permucocutaneously administered with such a desensitization agent in the form of a collyrium, collunarium or nebula for oral cavity. It was revealed that such an effect was more augmented when used a conjugate of the present protein and a saccharide which consists essentially of repeating maltotriose units such as pullulan and elsinan, and, in this case, the dose and administration period requisite for attaining the objective desensitization effect were more reduced or shortened as compared with other conjugates prepared by using saccharides other than pullulan and elsinan.

Experiment 3-4

Acute toxicity test

By using conventional method, mice, 20-day-old, were orally or intraperitoneally administered with therapeutic and/or prophylactic desensitization agents obtained by the method in Examples B-1 to B-4. As a result, it was revealed that the LD_{50} of the desensitization agents was 1,000,000 ng or more in any administration route. The results confirm that the present desensitization agent containing the present protein and a specific saccharide can be used in pharmaceuticals for administering to mammals including human without a fear of causing side effects.

As is described above, the protein according to the present invention is a novel substance causative of pollenosis. The protein has a feature to induce pollenosis in mammals including human, and because of this it is widely applicable to desensitization agents for diagnosing, treating and/or preventing pollenosis, as well as to diagnosis of pollenosis using enzyme immunoassay and radioimmunoassay, and to researches for elucidating the mechanisms of allergy induction in general. More particularly, the conjugates of the protein and a specific saccharide according to the present invention are characteristic in that they induce the production of immunoglobulin antibodies which are effective to desensitization when administered to mammals including human, and do not substantially form immunoglobulin E antibody which is a major factor causative of unsatisfactory side effects such as anaphylactic shock. Because of these properties, the present desensitization agent effectively reduces the dose of an antigen and even shortens the administration period requisite for the treatment and/or prevention of pollenosis. The protein with these useful properties can be readily prepared from cedar pollens as material in a satisfactory-high yield by the present process.

The present invention exerts the aforesaid outstanding effects and has a great significance and contribution to the field.

While there has been described what is at present considered to be the preferred embodiments of the invention, it will be understood the various modifications may be made therein, and it is intended to cover in the appended claims all such modifications as fall within the true spirits and scope of the invention.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:
(A) NAME: KABUSHIKI KAISHA HAYASHIBARA SEIBUTSU KAGAKU KENKYUJO
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(C) CITY: Okayama
(E) COUNTRY: Japan
(F) POSTAL CODE (ZIP): 700

(ii) TITLE OF INVENTION: PROTEIN, PROCESS TO PRODUCE THE SAME, AND USES THEREOF

(iii) NUMBER OF SEQUENCES: 5

(iv) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30 (EPO)

(vi) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: JP 347017/1993
(B) FILING DATE: 27-DEC-1993

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 6 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: unknown
(D) TOPOLOGY: unknown

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

Asp Asn Pro Ile Asp Ser
1 5

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 6 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: unknown
(D) TOPOLOGY: unknown

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

Ala Ile Asn Ile Phe Asn
1 5

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 23 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: unknown
(D) TOPOLOGY: unknown

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

Asn Gly Asn Ala Thr Pro Gln Leu Thr Lys Asn Ala Gly Val Leu Thr
1 5 10 15

Cys Ser Leu Ser Lys Arg Cys
20

(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 16 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: unknown
(D) TOPOLOGY: unknown

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION:9
(D) OTHER INFORMATION:/product= "OTHER"
/note= "X identified as Asn residue modified with sugar chain

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

Glu Ala Phe Asn Val Glu Asn Gly Xaa Ala Thr Pro Gln Leu Thr Lys
1 5 10 15

(2) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 41 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: unknown
(D) TOPOLOGY: unknown

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION:14
(D) OTHER INFORMATION:/product= "OTHER"
/note= "X identified as Asn residue modified with sugar chain

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

Thr Ala Thr Asn Ile Trp Ile Asp His Asn Ser Phe Ser Xaa Ser Ser
1 5 10 15

Asp Gly Leu Val Asp Val Thr Leu Thr Ser Thr Gly Val Thr Ile Ser
20 25 30

Asn Asn Leu Phe Phe Asn His His Lys
35 40

Claims

1. A protein having the following physicochemical properties:
(1) Molecular weight
44,000-54,000 daltons on sodium dodecyl polyacrylamide gel electrophoresis (SDS-PAGE);
(2) Isoelectric point (pI)
8.5-9.2 on isoelectrophoresis;
(3) Partial amino acid sequence containing the C-terminal
Possessing a partial amino acid, sequence containing the C-terminal as shown by

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                                                    -Asn-
Gly-Asn-Ala-Thr-Pro-Gln-Leu-Thr-Lys-Asn-
Ala-Gly-Val-Leu-Thr-Cys-Ser-Leu-Ser-Lys-
Arg-Cys;
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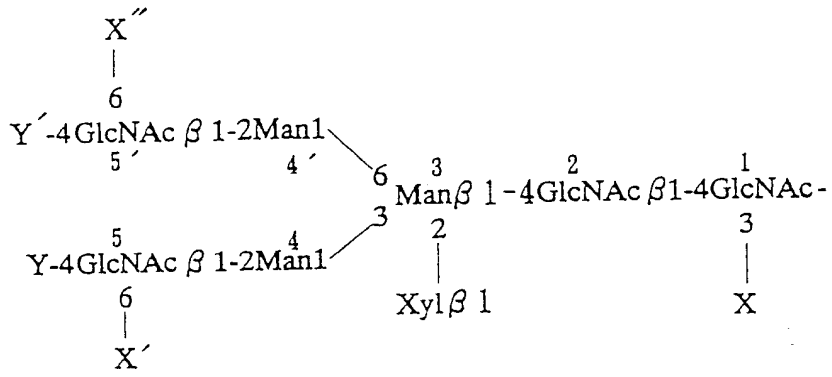
(4) Sugar content
Containing about 5-20% by weight of a sugar chain having a chemical structure in the molecule as shown by the following chemical formula. Where "X", "X'" and "X''" represent hydroxyl group or Fucα1, and "Y" and "Y'" represent hydroxyl group or Galβ1.

Chemical formula:



(5) Ultraviolet absorption spectrum
Exhibiting the maximum absorption spectrum at a wave length around 280 nm;
(6) Solubility in solvent
Soluble in water, physiological saline and phosphate buffer;
(7) Biological activity
Attaching to immunoglobulin E antibody collected from blood of a patient with pollenosis.
Inducing pollenosis; and
(8) Stability
Being inactivated in an aqueous solution (pH 7.2) when incubated at 100 °C for 10 minutes.
Substantially not losing its activity in an aqueous solution (pH 7.2) even when allowed to stand at 4 °C for one month.

2. The protein in accordance with claim 1, which is derived from a cedar pollen.

3. A process for preparing the protein of claim 1, which comprises extracting a cedar pollen with water or an aqueous solvent, and recovering the protein from the resultant extract.

4. The process in accordance with claim 3, wherein the recovering step comprises one or more methods selected from the group consisting of salting out, dialysis, filtration, concentration, centrifugation, gel filtration chromatography, ion-exchange chromatography, affinity chromatography, electrophoresis and isoelectrophoresis.

5. The process in accordance with claim 3 or claim 4, wherein said aqueous solvent is one or more members selected from the group consisting of aqueous solutions of methyl alcohol, ethyl alcohol and acetone.

6. A desensitization agent comprising a pharmaceutically-acceptable carrier and the protein of claim 1 or claim 2 as an effective ingredient.

7. The desensitization agent in accordance with claim 6, which contains as a stabilizer one or more members selected from the group consisting of serum albumin and gelatin.

8. The desensitization agent in accordance with claim 6 or claim 7, wherein the protein is covalently attached to a saccharide.

9. The desensitization agent in accordance with claim 8, wherein the saccharide is one or more members selected from the group consisting of starch, amylose, dextran, polysurose, pullulan, elsinan, curdlan, gum arabic, gum tragacanth, guar gum, xanthan gum, carrageenan, cellulose, glucomannan, chitosan, lipopolysaccharides, and their derivatives and partial hydrolysates.

10. The desensitization agent in accordance with claim 8 or claim 9, wherein the saccharide has an average molecular weight in the range of about 500-10,000,000 daltons.

11. The desensitization agent in accordance with any one of claims 8 to 10, wherein the weight ratio of the protein against the saccharide is about 1:0.001 to 1:1,000, on a dry solid basis.

12. The desensitization agent in accordance with any one of claims 8 to 11, wherein the covalent binding between the protein and the saccharide is formed by reacting them at a pH of about 0.1-12 and a temperature of about 0-100 °C for about 0.1-50 hours.

European Patent Office

EUROPEAN SEARCH REPORT

Application Number
EP 94 30 9386

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int.Cl.6)
X,D	EP-A-0 416 816 (KABUSHIKI KAISHA HAYASHIBARA SEIBUTSU KAGAKU KENKYUJO) * page 2, line 45 - page 10, line 28 * ---	1-12	C07K14/415 A61K39/36
X,D	EP-A-0 308 147 (KABUSHIKI KAISHA HAYASHIBARA SEBUTSU KAGAKU KENKYUJO) * page 2, line 49 - page 11, line 15 * ---	1-12	
Y	WO-A-93 01213 (IMMUNOLOGIC PHARMACEUTICAL CORPORATION) * page 9, line 9 - page 16, line 29 * ---	1,2,6	
Y	DATABASE WPI Section Ch, Week 8832 Derwent Publications Ltd., London, GB; Class B04, AN 88-224232 & JP-A-63 159 324 (LION CORP) , 2 July 1988 * abstract * ---	1-6	
Y,D	FEBS LETTERS, vol. 239,no. 2, November 1988 AMSTERDAM NL, pages 329-332, MADOKA TANIAI ET AL. 'N-TERMINAL AMINO ACID SEQUENCE OF A MAJOR ALLERGEN OF JAPANESE CEDAR POLLEN (CRY J I).' * the whole document * ---	1-6	TECHNICAL FIELDS SEARCHED (Int.Cl.6) C07K
Y,D	THE JOURNAL OF ALLERGY AND CLINICAL IMMUNOLOGY, vol. 71,no. 1, January 1993 pages 77-86, HIROSHI YASUEDA ET AL. 'ISOLATION AND PARTIAL CHARACTERIZATION OF THE MAJOR ALLERGEN FROM JAPANESE CEDAR (CRYPTOMERIA JAPONICA) POLLEN' * the whole document * -----	1-6	

The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
THE HAGUE	11 April 1995	Rempp, G

CATEGORY OF CITED DOCUMENTS

X : particularly relevant if taken alone
Y : particularly relevant if combined with another document of the same category
A : technological background
O : non-written disclosure
P : intermediate document

T : theory or principle underlying the invention
E : earlier patent document, but published on, or after the filing date
D : document cited in the application
L : document cited for other reasons

& : member of the same patent family, corresponding document

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